

THE OCCURRENCE OF IMMUNOGLOBULIN FRAGMENTS,
TWO TYPES OF LACTOFERRIN AND A LACTOFERRIN-IgG2
COMPLEX IN BOVINE COLOSTRAL AND MILK WHEY

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SUMMARY

When the fraction of bovine colostrum or normal whey soluble in 40% saturated $(\text{NH}_4)_2\text{SO}_4$ is applied to a column of DEAE-Sephadex A-50 equilibrated with 0.05 M NaCl, 0.1 M Tris, pH 8.3, the unadsorbed protein appears as three peaks (I, II, III). Each of these peaks can be subsequently resolved into 5-8 fractions by gel filtration. This fractionation procedure, combined with immunodiffusion and immunoelectrophoretic analyses, has allowed for characterization and identification of the proteins comprising the original three peaks obtained by DEAE chromatography. Peak I is predominately lactoferrin but IgG2-lactoferrin complexes and ribonuclease can also be detected. Peak II is predominately 7-S IgG2 while Peak III contains Fab, IgG2-Fc and an immunoelectrophoretically distinct form of lactoferrin. The secondary form of lactoferrin, present in purified lactoferrin preparations, appears to be of lower molecular weight and like lactoferrin, is antigenically distinct from the bovine milk protein lactollin. F(ab')_2 fragments are also present in this complex mixture while glycoprotein-a (free secretory component) is absent. The immunoelectrophoretic arcs of the IgG immunoglobulins described could explain the minor IgG-related precipitin arcs often observed in bovine serum and secretions without postulating a new subclass of IgG. The kinds of immunoglobulin fragments detected are similar to those observed in stored human serum and certain human exocrine secretions (except urine) and may result from proteolysis of intact IgG rather than *de novo* synthesis.

INTRODUCTION

Antisera to bovine immunoglobulins develop a number of minor immunoelectrophoretic arcs with particularly milk and colostrum whey and also, to a lesser extent, with serum. Such arcs have been reported by various investigators¹⁻⁵. Initial

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studies of such arcs allowed us to suggest that bovine glycoprotein-a was the secretory component for secretory IgA^{4,6,7}.

DEAE-cellulose^{1,8-10} and DEAE-Sephadex¹¹⁻¹⁵ have been used routinely for the preparation of bovine IgG immunoglobulins. When serum is fractionated on these ion exchangers, the protein not adsorbed on the column at low ionic strength is largely composed of IgG₂. When colostrum or milk whey is fractionated by the same procedure, the analogous protein fraction also contains lactoferrin^{7,15,17}, glycoprotein-a^{7,9,18}, ribonuclease⁷, and lactollin¹⁹. Using DEAE-Sephadex A-50, which also displays gel filtration properties, the unadsorbed proteins in colostrum or milk whey and occasionally serum are eluted in three fractions¹⁶. This paper reports the immunochemical and physicochemical identification of the proteins responsible for this multiphase pattern and suggests an explanation for a number of the minor immunoglobulin-related immunoelectrophoretic precipitin arcs. This report also presents evidence for the occurrence of two forms of lactoferrin and examines the antigenic relationship between lactoferrin and lactollin.

MATERIALS AND METHODS

Materials

Bovine colostrum was collected from Holstein-Friesian cows within 12 h after calving and stored at -20 °C prior to fractionation. This material and normal pooled herd milk was obtained from the Agricultural Research Center, Beltsville, Md. Normal or colostrum whey was prepared by removal of fat by centrifugation and by precipitation of casein with 0.1 M HCl¹⁶. Colostrum and normal whey were precipitated with 40% satd (NH₄)₂SO₄ to remove most of the immunoglobulins. The soluble fraction was dialyzed against the buffer used for DEAE-Sephadex chromatography.

Bovine IgG₁ and IgG₂ were prepared from colostrum and serum as previously described^{14,16}. Free secretory component was prepared from that fraction of milk whey insoluble in 50% satd (NH₄)₂SO₄ but soluble in 33% satd (NH₄)₂SO₄ by chromatography on DEAE-Sephadex followed by gel filtration. Free secretory component is eluted after the multiple fractions of unadsorbed proteins described in this paper^{7,16}. Total IgG (containing IgG₁ and IgG₂ in an approximate ratio of 3:2) was obtained by recycling gel filtration of that fraction of bovine serum insoluble in 33% satd (NH₄)₂SO₄. Purified bovine lactoferrin and lactollin were kindly provided by M. L. Groves.

Preparation of antisera

Antisera to total IgG, (IgG₁ plus IgG₂), free secretory component, IgG₁, lactollin and lactoferrin were prepared by immunizing normal rabbits and rabbits rendered tolerant to bovine IgG (ref. 20) with the appropriate proteins in Freund's complete adjuvant¹⁶. Antiserum to IgG₂ was raised in guinea pigs by the method of Binaghi *et al.*²¹. Antisera were rendered specific by absorption with insoluble immunoadsorbents prepared from IgG₂, IgG₁ and total IgG by the method of Avrameas and Ternynck²². Antiserum to bovine ribonuclease was kindly provided by E. J. Coulson. The specificity of these antisera is illustrated in Fig. 3. Figs 3A and 3B show that except for antisera to total IgG, free secretory component and lactollin, all antisera employed directly in this research are monospecific for their homologous antigens. The antiserum to lactollin also contains antibodies reactive with lactoferrin (Fig. 3A).

Antiserum to bovine IgM (μ -chains), although not used directly in this study, forms an additional precipitin line with 7-S IgM (ref. 16) (Fig. 3B). Antiserum to free secretory component demonstrates a reaction of partial identity with secretory IgA (ref. 7) (Fig. 3B).

The specificity of antiserum to total bovine IgG is complex and is illustrated in Fig. 3C. This antiserum detects determinants on IgG1 that are absent on IgG2; hence the small spur. The antiserum detects no difference between Fab (3.5 S) and F(ab')₂ (5 S) while IgG2 prominently spurs over each of these fragments.

Physicochemical methods

DEAE-Sephadex A-50 was equilibrated with 0.1 M Tris buffer containing 0.05 M NaCl, pH 8.3, and poured to form columns 20 cm \times 2.5 cm. The columns were further equilibrated by allowing buffer to flow through them overnight at 60 ml/h. Gel filtration was performed in a 2.5 cm \times 100 cm upward flowing column of Sephadex Superfine G-200 in 0.1 M Tris, 0.9 M NaCl, 0.003 M EDTA, 0.02% sodium azide, pH 8.4. Protein in the eluants from both ion-exchange and gel filtration columns was determined by the absorbance at 280 nm.

Molecular weight was estimated by gel filtration and sedimentation coefficients were determined in a Beckman Model E centrifuge at 52 640 rev./min in 0.01 M phosphate buffered saline (0.15 M), pH 7.1.

Preparation of digestion fragments

Total IgG was digested with mercuripapain (Worthington Biochemical Corp.) by the method of Porter²³ and the digest fractionated on Sephadex G-200. A fraction with an estimated molecular weight of 50 000 and sedimentation coefficient of 3.2 (6.5 mg/ml) was collected and the components identified as G1-Fc, G2-Fc and Fab on the basis of their immunoelectrophoretic behavior when compared to that previously reported for bovine¹¹ and human²⁴. Only Fab reacted with anti-light chain antiserum and G1-Fc and G2-Fc could be distinguished by reaction with their respective antisera.

Total IgG was hydrolyzed with crystallized pepsin (Worthington Biochemical Corp.) according to an established method²⁵. Five fractions were separated by gel filtration and corresponded in order of descending size to aggregated IgG, 7-S IgG, F(ab')₂, a trace of Fab and Fc and finally a mixture of peptides. The components were identified as described for the papain fragments. The presence of Fc-like material in pepsin digests of bovine IgG corroborates the data of Itsumi and Karush²⁶ for the rabbit. A 10 mg/ml sample of the F(ab')₂ fraction had a sedimentation coefficient of 5.0 and contained small amounts of 7-S material.

Immunochemical analyses

Microimmunoelectrophoresis was carried in 2% "Ionagar No. 2" according to the method of Scheidegger²⁷. Samples were electrophoresed for 90 min at 12 mA per slide over an ice bath employing a 0.05 M Veronal buffer, pH 8.1. Immunodiffusion was performed using standard micromethods²⁸.

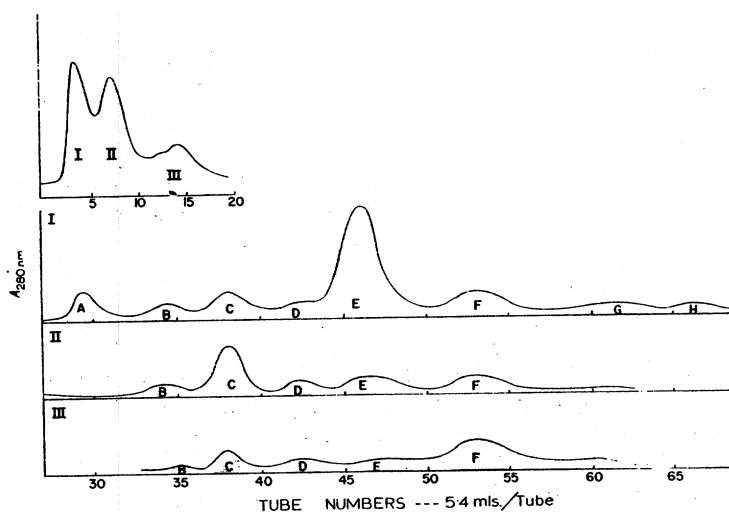


Fig. 1. (Upper Left) Elution profile from DEAE-Sephadex A-50 of the unadsorbed proteins in the 40% satd $(\text{NH}_4)_2\text{SO}_4$ soluble fraction of colostrum whey. (Center) Elution profiles obtained during gel filtration of DEAE Peaks I, II and III. Shaded areas represent the collected Fractions A-H.

RESULTS

The proteins in the 40% satd $(\text{NH}_4)_2\text{SO}_4$ soluble fraction of whey not adsorbed to DEAE-Sephadex, were resolved into Peaks I, II and III (Fig. 1). Each fraction was collected, concentrated and fractionated by gel filtration. Peak I could be resolved into eight fractions (A-H, Fig. 1) while Peaks II and III were resolved into slightly fewer fractions (Fig. 1). Preliminary immunoelectrophoretic analyses indicated that the corresponding B-F fractions separated from Peaks I, II and III were identical except for slight differences in the electrophoretic mobilities of the immunoglobulins present (Compare Fractions I-F and III-F, Fig. 4). Planimetric comparisons of the gel filtration elution profiles indicated that Fraction E comprised over 50% of Peak I, Peak C comprised nearly 40% of Peak II and Fraction F comprised nearly 50% of Peak III; these being the major fractions separated from each of the original DEAE peaks. The molecular weight of each fraction was estimated by gel filtration using bovine IgM, IgG, bovine serum albumin, β -lactoglobulin and α -lactalbumin as reference standards. These data as well as ultracentrifugational results are presented in Table I. All fractions except B and possibly H, contained some lactoferrin (Fig. 2A) and Fraction H contained ribonuclease (Fig. 2B). The precipitin pattern obtained with Fraction F, when tested with anti-lactoferrin, gave two parallel precipitin lines (Fig. 2A). When tested by immunodiffusion, Fractions A-F gave extremely complex patterns and it was necessary to use immunoelectrophoresis to resolve this complexity.

When analyzed by immunoelectrophoresis against antiserum to IgG and specific antiserum for IgG1, lactoferrin and free secretory component, the patterns shown in Fig. 4 were obtained. Single, but differing precipitin arcs were obtained with Fractions A-C and E using anti-IgG. Fractions D and F gave multiple arcs with this antiserum. IgG1 was primarily detected in Fraction C, the fraction containing the bulk of the 7-S

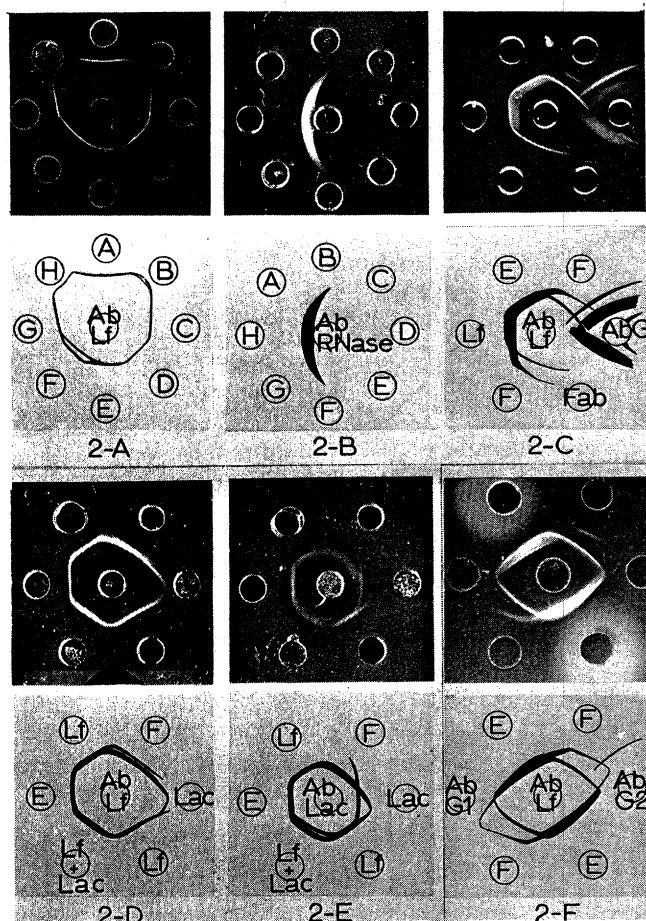


Fig. 2. Immunodiffusion analyses. A-H, gel filtration fractions (See Fig. 1); Lf, lactoferrin; Lac, lactollin; Fab, purified Fab fragment obtained by papain digestion of bovine total IgG; G, total IgG (IgG1 plus IgG2); G1, IgG1; G2, IgG2; RNase, bovine ribonuclease; Ab, preceding any of the above denotes antiserum prepared against that protein. Antiserum to lactollin also contains antibodies to lactoferrin.

IgG, although a faint arc could also be detected in Fraction D. The identity reaction between Fraction A and IgG2 (Fig. 3) identifies the IgG component as IgG2. The faint inside spur (Arc 1) in the IgG immunoelectrophoretic pattern (Fig. 4C) is equivalent to the faint spur of IgG1 over IgG2 (Fig. 3C) while the pronounced spurs obtained with this antiserum in Fig. 4D and in the 5-S pepsin digest (Arc 2 inside Arc 7) are equivalent to the prominent spur of IgG2 over $F(ab')_2$ or Fab (Fig. 3C). Free secretory component was absent from all fractions. A typical lactoferrin precipitin arc could be detected in Fractions D, E and F with a trace present in Fraction C. In addition, a prominent convex arc was observed in Fraction F which coalesced with the typical lactoferrin arc developed with this fraction. The same arc was also present, but less obvious, in Fraction E. Antiserum to lactoferrin also reacted strongly with

TABLE I

n.d., not determined.

Gel filtration fraction	Color	V_e (ml)*	Mol. wt estimated by gel filtration [‡]	$s_{20,w}^{**}$	Principal constituent identified
A	pink	157	700 000+	n.d.	Lactoferrin-IgG2 Complex
B	colorless	186	330 000	9.8 [†] + 7.1	IgG2 dimers
C	colorless	205	170 000	6.7	7-S IgG2
D	colorless	230	115 000	5.2 [†] + 6.9	F(ab') ₂
E	red	252	72 000	4.8	Lactoferrin
F	pink	286	45 000	3.1-3.5	IgG2-Fc, Fab and low mol. wt lactoferrin
G	colorless	335	23 000	n.d.	Unknown
H	colorless	367	15 000	n.d.	Ribonuclease

* Elution volume of G-200 column measured to peak of eluted fraction. Void volume of the column was 150 ml. Values are based on the average of the values obtained with the three DEAE Peaks I, II, and III.

** Not extrapolated to zero concentration, concentrations ranged from 3 to 8 mg/ml.

† Largest peak.

‡ As all fractions except possibly C, E and H contained more than one component the determined mol. wt cannot be directly assigned to the constituent proteins.

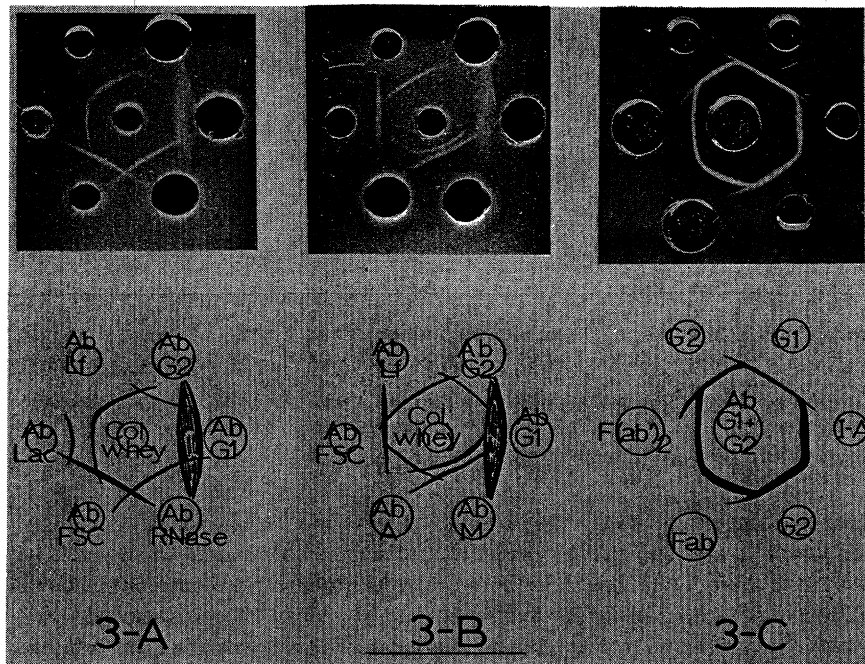


Fig. 3. Antigenic specificity of antisera employed. A and B, Antisera (Ab) to IgG1, IgG2, ribonuclease, lactoferrin, free secretory component (FSC), IgM μ -chains (M) and IgA α -chains (A) tested against bovine colostrum whey (Col. Whey). Same legend as Fig. 2 except as indicated above. The diffuse precipitin line obtained with antiserum to IgG1 results from the extremely high concentration of this protein in bovine colostrum whey. C, Specificity of antiserum to total IgG (Indicated as "a" in Fig. 4). The Fab and F(ab')₂ fractions were obtained as described in the text. I-A, Gel filtration fraction designated as such in Fig. 1.

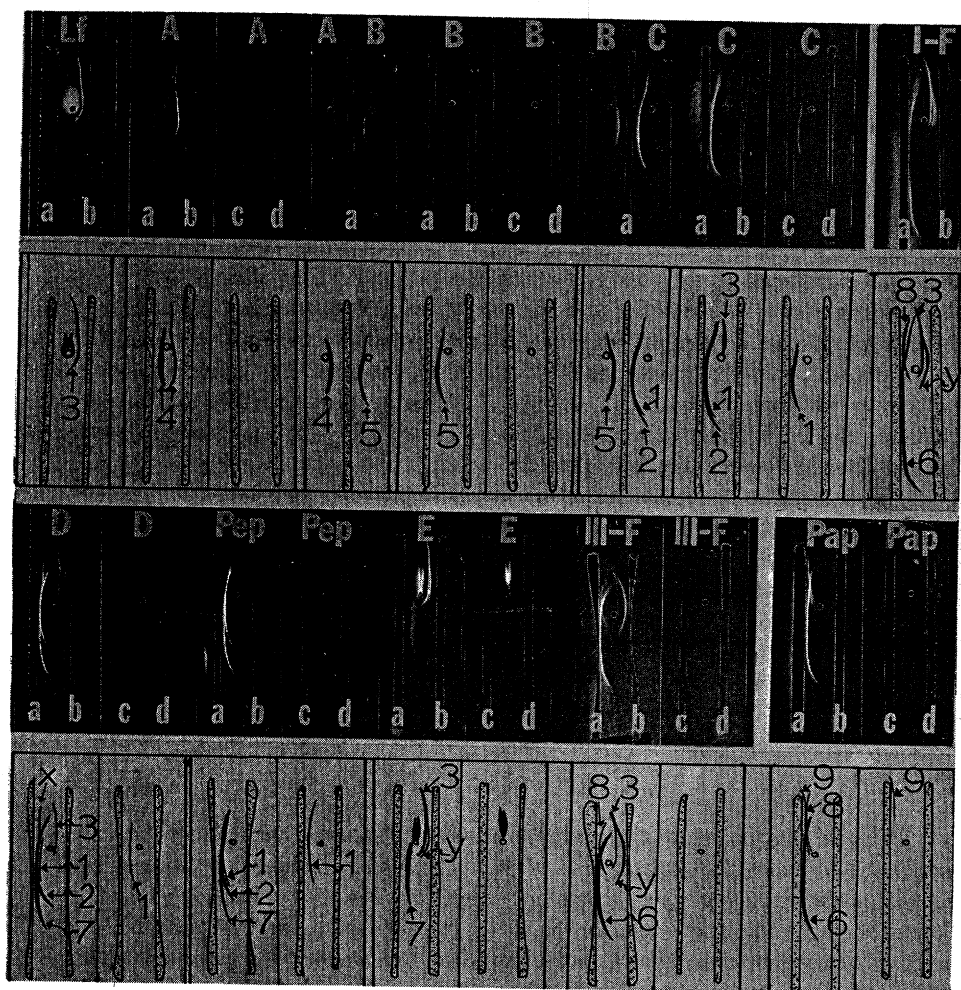


Fig. 4. Immunoelectrophoretic analyses of gel filtration fractions A-F, lactoferrin (Lf) and pepsin and papain digestion fragments. Anode at the top. Each photo is accompanied by a corresponding sketch. A-F gel filtration fractions (See Fig. 1); a, antiserum to IgG1 plus IgG2; b, antiserum to lactoferrin; c, antiserum to IgG1; d, antiserum to bovine free secretory component; Pep, 5-S fraction of pepsin digest of total IgG; Pap, 3.2-S fraction of papain digest of total IgG. I-F and III-F = corresponding Fractions F from DEAE Peaks I and III. Note difference in electrophoretic mobility of Fab fragment between these fractions. The following arcs are identified on the sketch: 1, IgG1; 2, IgG2; 3, lactoferrin; 4, lactoferrin-IgG2 aggregate; 5, IgG2 dimer; 6, Fab; 7, F(ab)₂; 8, IgG2-Fc; 9, IgG1-Fc; X, unidentified fragment; Y, lactoferrin related protein.

Fraction A, but in this case, the cathodal end of the lactoferrin arc paralleled that of the arc developed with anti-IgG in the same fraction.

When the F(ab')₂ material obtained by digestion with pepsin was analyzed by immunoelectrophoresis, the pattern obtained with anti-IgG was almost identical to that obtained with Fraction D using the same antiserum (Fig. 4). Similarly, when the Fc + Fab-rich fraction obtained with papain was tested with this antisera, the immunoelectrophoretic pattern was similar to that in Fraction F except only a single

Fc precipitin arc was present in Fraction F (Fig. 2). $F(ab')_2$ and Fab are identified in this study by the estimated molecular weights and sedimentation coefficients of the digestion fractions and elution Fractions D and F (Fig. 1) because antiserum to total IgG failed to distinguish these fragments antigenically (Fig. 3C).

The pattern of the partially coalescing lactoferrin arc in Fraction F (Arc Y) was similar to that identified as Fc using anti-IgG to test the same fraction (Arc 8). To determine if the lactoferrin arc resulted from an Fc-lactoferrin complex, the immunodiffusion tests shown in Figs 2C and 2F were set up. It can be seen that Fraction F \times anti-IgG or anti-IgG2 precipitin lines cross the precipitin lines obtained as a result of the reaction between Fraction F and anti-lactoferrin. The inner lactoferrin line must correspond to Arc Y seen on immunoelectrophoresis. Consistent with the immunoelectrophoretic pattern, some Arc Y is also visible in Fraction E and in the purified lactoferrin obtained from M. L. Groves. The same immunodiffusion test (Fig. 2F) identifies the Fc fragment as IgG2-Fc and not IgG1-Fc. This is also consistent with the immunoelectrophoretic data (Fig. 4).

To determine the relationship between the lactoferrin related protein in Fraction F (Arc Y) and the bovine milk protein lactollin, the immunodiffusion test in Fig. 2D was performed. No precipitin line was developed with lactollin. Reciprocally, antiserum raised against lactollin developed precipitin lines with the lactoferrin containing preparations (Fig. 2E and 3A). The lactollin precipitin line spurred over the lactoferrin arc developed in Fraction F (Fig. 2E) indicating that lactollin is antigenically distinct from lactoferrin and the lactoferrin-related component in Fraction F.

DISCUSSION

The similar molecular size and immunoelectrophoretic behavior of the fragments found in whey and those obtained by pepsin and papain digestion using antisera of defined specificity (Fig. 3) supports the identification given them (Fig. 4, Table I). Consistent with the known elution behavior of the bovine immunoglobulins^{1,4,10-12,16} most all of the immunoglobulins in the multiple-unadsorbed fractions are IgG2. Only traces of IgG1 were detected (Fig. 4). Although the Fab and $F(ab')_2$ fragments identified could be derived from either bovine subclass, only IgG2-Fc was detected (Fig. 2F). This should not be construed to mean that only fragmentation of IgG2 occurs, as an elution gradient designed to remove the protein adsorbed on the DEAE-Sephadex column did reveal traces of IgG1-Fc, as would be predicted on the basis of their greater net negative charge (Fig. 4, Pap.)

The presence of IgG fragments or microimmunoglobulins in external secretions is best known from studies on urine and the history of their discovery is reviewed elsewhere²⁹. Most common of the urinary fragments is not Fab but proteins related to the Fc-fragment, especially Fc' fragments^{30,31}. The latter apparently arise by degradation of urinary Fc rather than intact IgG (ref. 31). Urinary Fc may in turn be derived directly from the Fc found in plasma³² and this material is probably not simply derived from proteolytic activity³³ but its occurrence may be related to heavy chain disease³⁴. The occurrence of Fab and $F(ab')_2$ as well as Fc fragments is well known in other secretions and they are probably derived primarily from proteolytic activity³⁵. The fragments described in this report are most likely derived by proteolysis and

they accordingly resemble most closely the immunoglobulin fragments resulting from storage of serum³⁶. On two occasions during the isolation of serum IgG₂, IgG-related proteins were eluted from DEAE-Sephadex at the same molarity as the fragments described here. Their identical immunoelectrophoretic pattern suggested they were Fab, F(ab')₂ and Fc fragments⁷.

Although there is some evidence for covalently bonded IgG₁ dimers in bovine colostrum whey⁷, there is no evidence that the IgG₂ dimers reported here are more than hydrophobic polymers resulting from the low salt conditions. Similarly, the IgG₂-lactoferrin complex demonstrated may have the same origin as there are numerous reports in the literature attesting to the "stickiness" of lactoferrin^{15,19,37-40}. It has been shown that lactoferrin^{41,42} and serum transferrin⁴³⁻⁴⁶ can inhibit microbial growth by a derangement of iron metabolism. Bullins *et al.*⁴³ demonstrated that mixtures of transferrin and γ -globulin closely reproduced the same inhibitory effect on the growth of *Colostridium welchii* as was obtained with whole serum. Similarly, Szilagyi *et al.*⁴⁶ found that the α 2- and γ 2-globulin fractions of serum were most effective in inhibiting the growth of *Cryptococcus neoformans*. It is possible that the complexing of lactoferrin to γ -globulin represent a mechanism of specific humoral immunity.

The precipitin arcs formed by the lactoferrin-IgG₂ complex and IgG₂ dimers differ from those of γ -S IgG₂. We have previously demonstrated that when mixed, separate precipitin arcs are obtained for each which do not join¹⁶.

The molecular weight reported here for the lactoferrin in Fraction E (Table I) is close to that reported by others^{15,37,39}. Because the molecular weight estimate of Fraction F results in part from the presence of Fab and Fc fragments, the molecular weight of the lactoferrin related protein in this fraction cannot be ascertained. Its prominence in Fraction F rather than E suggests a molecular weight less than normal lactoferrin. Such a protein has not been previously reported. Studies of human milk do suggest the existence of other proteins that are antigenically related to lactoferrin (B. Tomasi, personal communication). A crystalline protein, lactollin, was isolated from bovine milk and colostrum and is reported to have a mol. wt of 43 000 (ref. 47). As demonstrated, this protein appears antigenically distinct from both the normal and the apparent low molecular weight lactoferrin reported here. In addition, lactollin is almost insoluble at pH 8.0 whereas the new lactoferrin-related protein reported here exhibits no such characteristics. Preliminary C-terminal analyses of lactollin and lactoferrin reveal no similarities (M. L. Groves and E. Kalan, personal communication). The presence of antibodies to lactoferrin in the lactollin antiserum can be explained by contamination resulting from the previously described stickiness of highly antigenic lactoferrin and the low immunogenicity of the poorly soluble lactollin. There are sufficient data which have shown that lactoferrin is a single polypeptide chain (refs 37 and 48 and M. L. Groves, personal communication). Therefore, the new lactoferrin-related component described here could represent either (1) a proteolytic digestion fragment of lactoferrin, or (2) a separate but related gene product but not a polypeptide chain subunit resulting from reduction of lactoferrin.

The immunoelectrophoretic arcs resulting from the reaction of anti-IgG and the various IgG fragments and aggregates described provide one explanation for the "intermediate" IgG precipitin arc often observed^{1,3,4} without postulating the existence of an additional bovine IgG subclass. Similarly, the detection of a second form of

lactoferrin helps to explain the discontinuous distribution of lactoferrin observed in this laboratory and by others (ref. 49 and B. Tomasi, personal communication) during ion-exchange fractionation of human and bovine milk whey.

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